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Tokyo 208 (JP)**(54) METHOD OF ASSAYING LIMULUS REAGENT-REACTIVE SUBSTANCE**

(57) For the purpose of selecting the wavelengths at which a highly sensitive and highly accurate determination is possible without being affected by interfering substances in the determination of a limulus reagent reactive substances by means of turbidimetric limulus test, in a method of determining a limulus reagent reactive substance (endotoxin or (1→3)- $\beta$ -D-glucan) in a sample by reacting the limulus reagent with the sample solution to lead to gelation followed by determining the change in the absorbance of transmitted light based on the increase in the turbidity of the reaction solution in process of the gelation, two different types of light, one of which is light with a test wavelength from 460 to 550 nm and the other of which is light with a reference wavelength from 650 to 800 nm, are employed as the light to irradiate the reaction solution and then the difference in absorbance value between the two types of light is determined and correlated to the concentration of the limulus reagent reactive substance.

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**Description****Field of the Invention**

The present invention relates to a method of determining a limulus reagent reactive substance such as endotoxin and (1→3)- $\beta$ -D-glucan (hereinafter referred simply to as  $\beta$ -glucan) using a horseshoe crab amebocyte lysate reagent (hereinafter referred to as limulus reagent).

**Background of the Invention**

Since adverse reactions such as severe fever and shock may develop when a blood, transfusion fluid or solution for injection contaminated with endotoxin is infused into the human body, the content of endotoxin should be determined in the process of the manufacturing of any pharmaceutical formulations such as transfusion fluid and solution for injection in order to prevent them from contamination by endotoxin.

It was found that horseshoe crab amebocyte lysate reacts with an endotoxin to lead to gelation, and a method using this phenomenon has conventionally been applied as an alternative to the fever test in rabbits to determine the endotoxin.

In this method, for the purpose of evaluating objectively the degree of turbidity of the reaction solution (reaction mixture containing a sample solution and limulus reagent) after gelation, a certain light is irradiated to the reaction solution and the decrease of quantity of transmitted light as a time goes on (increase in absorbance) is detected to obtain the ratio based on the initial value, and the time taken for specimens to reach a particular threshold value is regarded as the gelation period, which is employed as an index in the determination of the endotoxin (a type of turbidimetric limulus test using the reaction rate method (kinetic method); See Turbidimetric time analysis; examined published Japanese patent application 5-31744).

In such an optical determination method, a light-emitting diode which emits a light with a wavelength of about 660 nm is employed as a light source and a photo diode or photoelectric cell is employed as a detector for quantifying the light.

$\beta$ -glucan is also known to lead horseshoe crab amebocyte lysate to gelation.

In such conventional optical determination methods, it has not been considered whether the wavelength of the light emitted from the light source is suitable or not for the detection of the transmittance or absorbance of the reaction solution in the process of the gelation. In addition, a conventional turbidimetric limulus test employs determination using only the light with a single wavelength.

The determination should also be conducted with a stable detection value and a sensitivity which is practically allowable even if the sample solution contains interfering substances such as colored pigments, pro-

teins and nucleic acids. However, none of the conventional methods fulfil such requirements.

Accordingly, the method of determination of the present invention is designed for the purpose of selecting the two different wavelengths of light (test wavelength and reference wavelength) which enable a highly sensitive and highly accurate determination without being affected by interfering substances when limulus reagent reactive substances such as endotoxin and  $\beta$ -glucan are determined by means of a turbidimetric limulus test.

**Disclosure of the Invention**

15 The method of determining a limulus reagent reactive substance according to the present invention is a method of determining a limulus reagent reactive substance in a sample by reacting the limulus reagent with the sample solution to lead to gelation followed by determining the change in the absorbance of the transmitted light based on the increase in the turbidity of the reaction solution in process of the gelation, characterized in that different two types of light, one of which is the light with a test wavelength from 460 to 550 nm and the other 20 of which is the light with a reference wavelength from 650 to 800 nm, are employed as the light with which to irradiate the reaction solution and then the difference in the absorbance value between the two types of light is determined and correlated with the concentration of the limulus reagent reactive substance.

As the limulus reagent reactive substance, an endotoxin or (1→3)- $\beta$ -D-glucan may be considered.

**Brief Description of the Drawings**

35 Figure 1 shows a side view of an example of the turbidity determination device used in the method of determining a limulus reagent reactive substance according to the present invention.

40 Figure 2 shows a graph of a specific curve representing the relationship between the reference wavelength and the difference in absorbance in the determination using the two wavelengths ((a) is the ratio of the difference in absorbance, and (b) is the difference in absorbance) at a test wavelength of 490 nm.

45 Figure 3 shows a graph of a specific curve representing the relationship between the reference wavelength and the difference in absorbance in the determination using the two wavelengths ((a) is the ratio of the difference in absorbance, and (b) is the difference in absorbance) at a test wavelength of 520 nm.

50 Figure 4 shows a graph of a specific curve representing the relationship among the rate of change in difference in absorbance of the reaction solutions and the concentration of the limulus reagent reactive substance (endotoxin).

55 Figure 5 shows a graph of a specific curve representing the relationship between the time required until the absorbance of the reaction solution reaches a cer-

tain threshold and the concentration of the limulus reagent reactive substance (endotoxin).

Figure 6 shows a graph of a specific curve representing the relationship between the time required until the absorbance of the reaction solution reaches a certain threshold and the concentration of the limulus reagent reactive substance ( $\beta$ -glucan).

#### Preferred Embodiments of the Present Invention

The limulus reagent employed in the present invention is a blood-cell extract (horseshoe crab amebocyte lysate) or processed materials thereof, which is prepared by a usual method (See, for example, Journal of Biochemistry, 80, 1011-1021 (1976)) from hemolymph of horseshoe crabs such as Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas, Carcinoscorpius rotundicauda and the like, and is a reagent which reacts with endotoxin and/or  $\beta$ -glucan whereby leading to gelation.

The processed materials mentioned above are, for example, those obtained by extracting the horseshoe crab amebocyte lysate with an organic solvent such as chloroform or by adding a surfactant to enhance the sensitivity to the endotoxin. While the horseshoe crab amebocyte lysate usually contains both, endotoxin sensitive factor (factor C) and  $\beta$ -glucan sensitive factor (factor G), the processed materials mentioned above have been subjected to the fractionation or removal of either factor C or factor G through the treatment of the horseshoe crab amebocyte lysate with dextran sulfate or sulfopropyl group-carrying supports so that the reaction occurs exclusively with either the endotoxin or  $\beta$ -glucan.

In addition, the processed materials also include those which have been processed by preparing the horseshoe crab amebocyte lysate in the presence of the polyglycoside in which a specific number of (1 $\rightarrow$ 3)- $\beta$ -D-glucoside structural units are linked in order to inhibit the activation of factor G whereby achieving an exclusive reaction with the endotoxin. The limulus reagent may be in the form of a liquid, powder or solid.

In the present invention, the sample solutions to be examined for the presence of the limulus reagent reactive substances are not specifically limited and may include blood, urine, cerebrospinal fluid, transfusion fluid, solution for injection, water and the like.

A sample solution is pretreated appropriately with acid, alkali, heating, surfactant and the like and reacted with a limulus reagent in a suitable reaction vessel to lead to the gelation; and the increase in turbidity in process of the gelation is determined by irradiating the reaction solution with the two types of light having different wavelengths (test wavelength of from 460 to 550 nm and reference wavelength of from 650 to 800 nm) to obtain absorbance of each, from which the difference in the absorbance between both types of light is calculated. Then, the solutions containing the limulus reagent reactive substance with known concentrations are subjected to similar determination to obtain a calibration

curve, to which the value of the sample solution is compared, whereby quantifying the limulus reagent reactive substance in the sample solution. The calculation of the difference in the absorbance and the comparison of the calibration curve with the value of the sample determined may be conducted automatically using previously designed programs.

To react the limulus reagent with the sample solution, the sample solution is mixed with the limulus reagent to form a reaction solution and the reaction solution is reacted at 30 to 50°C, preferably at 35 to 45°C for 15 minutes to 3 hours, preferably for 30 to 90 minutes.

The absorbance can be determined after the termination of the reaction (end-point method) or during the reaction (reaction rate method; kinetic method). In the kinetic method, the time required until the absorbance (transmittance) of the reaction solution reaches a certain value is regarded as the gelation time (turbidimetric time analysis) or the rate of the change in the absorbance of the reaction solution may be determined.

The reaction vessel may be any of those used in usual limulus tests, provided that the material and shape of the vessel do not substantially affect the transmission of the light. For example, a glass or plastic tube or a microplate is preferable.

An example of the absorbance determination device employed in an embodiment of the present invention, in cases where a microplate is used as the reaction vessel, is provided with an optical system consisting of, as shown in Figure 1, a microplate 1 for determination which is mounted on a carrier 5 and provided with a plural of wells 11 which are to contain reaction solution 14 consisting of the sample and the reagent, a light source 3 consisting of two filters 33a and 33b through which the lights with specific wavelengths selected via a luminescent body 31 such as a bulb, a slit 32 and a rotating device 35 such as a motor are transmitted, optical fibers 34 which resolves the light selected via the two filters 33a and 33b into the number of lines of wells 11 in the microplate 1 for determination, and photoelectric conversion elements 4 for converting strength of the light transmitted through the reaction solution 14 into electric signal. An aluminum plate 2 having holes 21 provided in the bottom of microplate 1 is for the establishing of a uniform distribution of the temperature.

#### Embodiment 1: Determination of endotoxin by end-point method

Using a method of determining a limulus reagent reactive substance according to the present invention, the following experiment was conducted to select the appropriate wavelengths of the lights which are not affected by interfering substances such as proteins and colored pigments which may be present in a sample solution.

1 ml of a commercial limulus reagent (manufactured by Associates of Cape Cod, marketed by Seika-

gaku Corporation under the trade name of "Pyrotell") was placed in a glass tube, to which 1 ml of the solution of *E. coli* strain 0111:B4-derived endotoxin (0.25 EU/ml, "EU" stands for endotoxin unit) was added, reacted at 37°C for 30 minutes and then quenched with 0.02 ml of 10% trifluoroacetic acid.

Subsequently, in the following conditions:

- condition A, wherein to the reaction mixture described above 25% human serum albumin (HSA) formulation (Green Cross Corp.) was added as a protein to the final concentration of 0.5%;
- condition B, wherein to the reaction mixture described above 5mM paranitroaniline (pNA) was added as a yellow pigment to the final concentration of 0.5mM;
- condition C, wherein to the reaction mixture described above distilled water (DW) was added in the volume equal to those of the aqueous solution in the conditions A and B;

three sample solutions were prepared and irradiated using light with a test wavelength ranging from 200 to 890 nm and with a reference wavelength ranging from 210 to 900 nm stepwise at 10-nm intervals, and the absorbance at each wavelength was determined to calculate the difference in the absorbance between both wavelengths. Then, the combination of the test wavelength and the reference wavelength at which the ratio of the difference in the absorbance between the two wavelengths under the condition A (HSA) or the condition B (pNA) to the difference in the absorbance between the two wavelengths under the condition C (DW) is  $1.0 \pm 0.05$  and at the same time the difference in the absorbance between the two wavelengths under each condition is 0.2 or more was analyzed. As a result, the combination of a test wavelength of from 460 to 550 nm and a reference wavelength of from 650 to 800 nm was selected.

As an example, the results of the analysis of the data obtained by subtracting the absorbance at each reference wavelength ranging from 500 to 900 nm from the absorbance at the test wavelength of 490 nm are shown in Figures 2 (a) and (b).

Thus, in Figure 2 (a) in which the abscissa represents the reference wavelength (500 to 900 nm), the specific curve on the solid circles represents the ratio (B/A) of "the difference (B) in the absorbance between the two wavelengths using the solution of endotoxin dissolved in the aqueous solution of paranitroaniline (pNA)" to "the difference (A) in the absorbance between the two wavelengths using an aqueous solution of the endotoxin", while the specific curve on the open circles represents the ratio (C/A) of "the difference (C) in the absorbance between the two wavelengths using the solution of endotoxin dissolved in the human serum albumin (HSA)" to "the difference (A) in the absorbance between the two wavelengths using an aqueous solution of the endotoxin" as the ordinate.

In Figure 2 (b), the specific curve on the open triangles represents "the difference (A) in the absorbance between the two wavelengths using an aqueous solution of the endotoxin" as the ordinate, the specific curve on the solid circles represents "the difference (B) in the absorbance between the two wavelengths using the solution of endotoxin dissolved in the aqueous solution of paranitroaniline (pNA)" as the ordinate, and the specific curve on the open circles represents "the difference (C) in the absorbance between the two wavelengths using the solution of endotoxin dissolved in the human serum albumin (HSA)" as the ordinate.

Similarly, for these three reaction solutions, the results of the analysis of the data obtained by subtracting the absorbance at each reference wavelength ranging from 530 to 900 nm from the absorbance at the test wavelength of 520 nm are shown in Figures 3 (a) and (b).

Based on these results, the ranges of the test and reference wavelengths which gave the results showing almost no difference from the distilled water (condition C: DW) (i.e. the ratio of the difference between the two wavelength was almost 1) under both of the conditions in which the protein was present (condition A: HSA) and in which the pigment was present (condition B: pNA) while giving higher absorbance values were found to be from 460 to 550 nm and from 650 to 800 nm, respectively.

### Embodiment 2: Determination of endotoxin by the kinetic method

0.05 ml of "Pyrotell" was dispensed to a 96-well microplate (Toxipet plate 96F, Seikagaku Corporation), to which each 0.05 ml of the solutions of *E. coli* UKT-B-derived endotoxin at 5 different concentrations within the range from 0.005 to 100 EU/ml was then added. The microplate was subjected to the following conditions:

- condition A1, wherein 25% human serum albumin (HSA) formulation was added as a protein to a final concentration of 0.5%;
- condition A2, wherein 25% human serum albumin (HSA) formulation was added as a protein to a final concentration of 0.1%;
- condition B, wherein 5mM para-nitroaniline (pNA) was added as a yellow pigment to a final concentration of 0.5mM;
- condition C, wherein distilled water (DW) was added in a volume equal to those of the aqueous solutions in the conditions A1, A2 and B;

to prepare 20 different reaction solutions (= 5 concentrations x 4 conditions), which were dispensed to each well of the microplate, which was then placed in a microplate reader fitted with a thermostat (Wellreader SK 601, Seikagaku Corporation). After mixing, the solutions were allowed to react at 37°C while irradiating light A with the test wavelength of 490 nm and light B with the

reference wavelength of 660 nm at 15 seconds interval, whereby determining the absorbance (a, b) of the two lights A and B having different wavelengths and calculating the difference in the absorbance (a-b) between the two wavelengths for each reaction solution.

By logarithmically plotting the rate of change in the difference in the absorbance (a-b) mentioned above against the concentration of the endotoxin, the specific curve shown in Figure 4 was obtained.

Based on the specific curve shown in Figure 4, values almost equal to those of the distilled water (condition C: DW) were obtained even in the presence of the protein (condition A1, A2: HSA) or the yellow pigment (condition B: pNA) and at the same time the rate of change in the difference in the absorbance (a-b) was in proportion to the concentration of the endotoxin, indicating satisfactory linearity and reproducibility even in the presence of the interfering substances.

In addition, by logarithmically plotting the time period (gelation time, t) required until the difference in the absorbance (a-b) reached a certain threshold (absorbance of 0.006) against the endotoxin concentration, the specific curve shown in Figure 5 was obtained.

Based on the specific curve shown in Figure 5, values almost equal to those of the distilled water (condition C: DW) were obtained even in the presence of the protein (condition A1, A2: HSA) or the yellow pigment (condition B: pNA) and, at the same time, the time period t required until the difference in the absorbance (a-b) reached a certain threshold was inversely proportional to the concentration of the endotoxin, indicating satisfactory linearity and reproducibility even in the presence of the interfering substances.

Under conditions selected based on the results of the determination in the embodiment 1 (test wavelength: 460 to 550 nm, reference wavelength: 650 to 800 nm), the change in turbidity practically using the endotoxin under the conditions A1, A2, B and C mentioned above was analyzed based on the kinetic method, and the calibration curve vs. the endotoxin concentration was made at all combinations of the two different wavelengths. As a result, it was proven that the stable determination of the turbidity was possible even in the presence of any of the two interfering substances, i.e., the protein (condition A1, A2) or the pigment (condition B).

#### Embodiment 3: Determination of $\beta$ -glucan concentration by kinetic method

0.05 ml of a commercial limulus reagent (manufactured by Associates of Cape Cod, marketed by Seikagaku Corporation under the trade name of "Pyrotell-T") was placed in the wells of Toxitest plate 96F, to which each 0.05 ml of pachymann solution as  $\beta$ -glucan (dissolved in 0.1N NaOH and then diluted with DW) was added at any of the three different concentrations within the range from 0.01 to 1.0 ng/ml. The mixture thus obtained was subjected to the following conditions:

- 5 - condition A, wherein 25% human serum albumin (HSA) formulation was added as a protein to the final concentration of 0.5%;
- condition B, wherein 5mM para-nitroaniline (pNA) was added as a yellow pigment to the final concentration of 0.5mM;
- condition C, wherein distilled water (DW) was added in a volume equal to those of the aqueous solutions in the conditions A and B;

10 to prepare 9 different reaction solutions (= 3 concentrations  $\times$  3 conditions), which were processed in a manner similar to that for the embodiment 2 except that a test wavelength of 500 nm and a reference wavelength of 700 nm were used.

15 Then, by logarithmically plotting the time period (gelation time, t) required until the difference in absorbance reached a certain threshold against the concentration of  $\beta$ -glucan, the specific curve shown in Figure 6 was obtained.

20 Based on the specific curve shown in Figure 6, also in the determination of  $\beta$ -glucan, it was shown that satisfactory linearity and reproducibility were obtained without any effect from the presence of the interfering substances.

#### Industrial Applicability

25 As is evident from the description in the embodiments, in a method of determining a limulus reagent reactive substance according to the present invention which employs a turbidimetric limulus test using an endotoxin or  $\beta$ -glucan as a limulus reagent reactive substance, a 2-wavelength determination method in which a test wavelength of 460 to 550 nm and a reference wavelength of 650 to 800 nm are selected enables the prevention of the physical interference (strain, scratch, etc.) of the reaction vessel such as microplates, and thereby the stable, highly sensitive and highly accurate determination without any effect from contaminating proteins or colored pigments becomes possible.

#### Claims

- 30 1. A method of determining a limulus reagent reactive substance in a sample by reacting the limulus reagent with the sample solution to lead to gelation followed by determining the change in the absorbance of transmitted light based on the increase in the turbidity of the reaction solution in process of the gelation, characterized in that two different types of light, one of which is light with a test wavelength of from 460 to 550 nm and the other of which is light with a reference wavelength of from 650 to 800 nm, are employed as the light to irradiate the reaction solution and then the difference in the absorbance value between the two types of light is determined and correlated to the concentration of the limulus reagent reactive substance.
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2. A method of determining a limulus reagent reactive substance according to claim 1 wherein the limulus reagent reactive substance is an endotoxin or (1→3)- $\beta$ -D-glucan.

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Figure 1

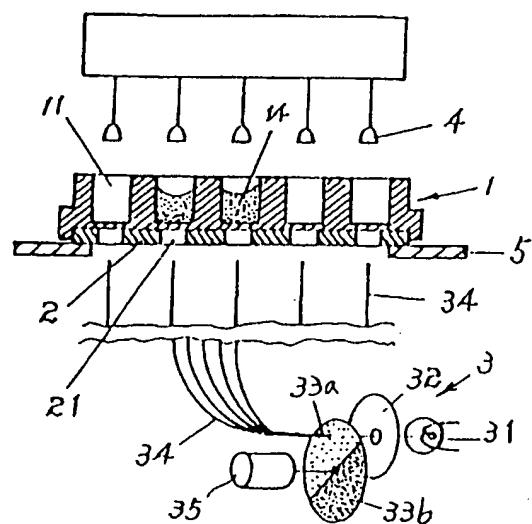


Figure 4

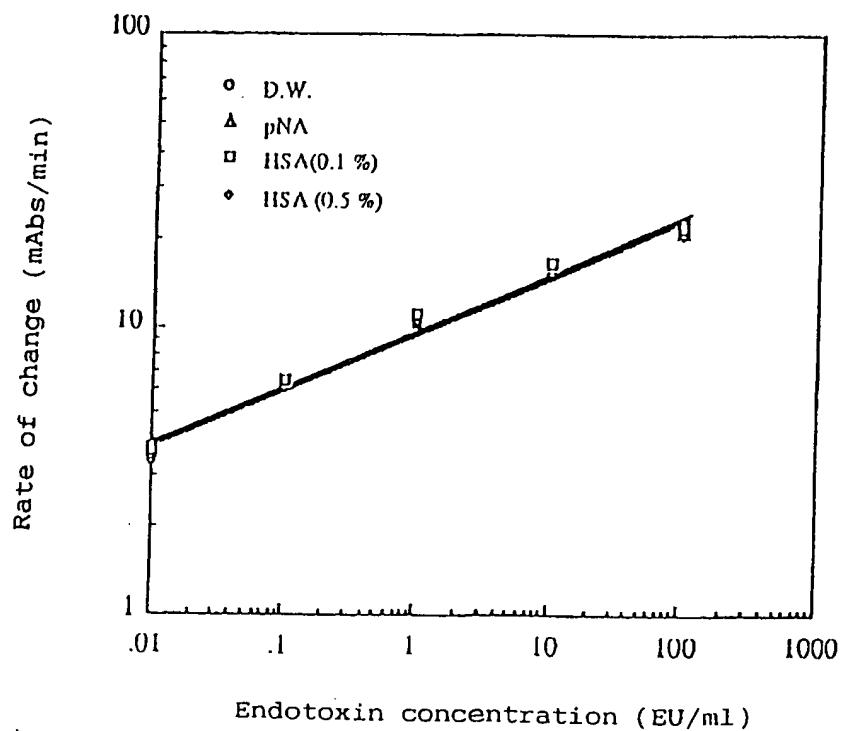


Figure 2

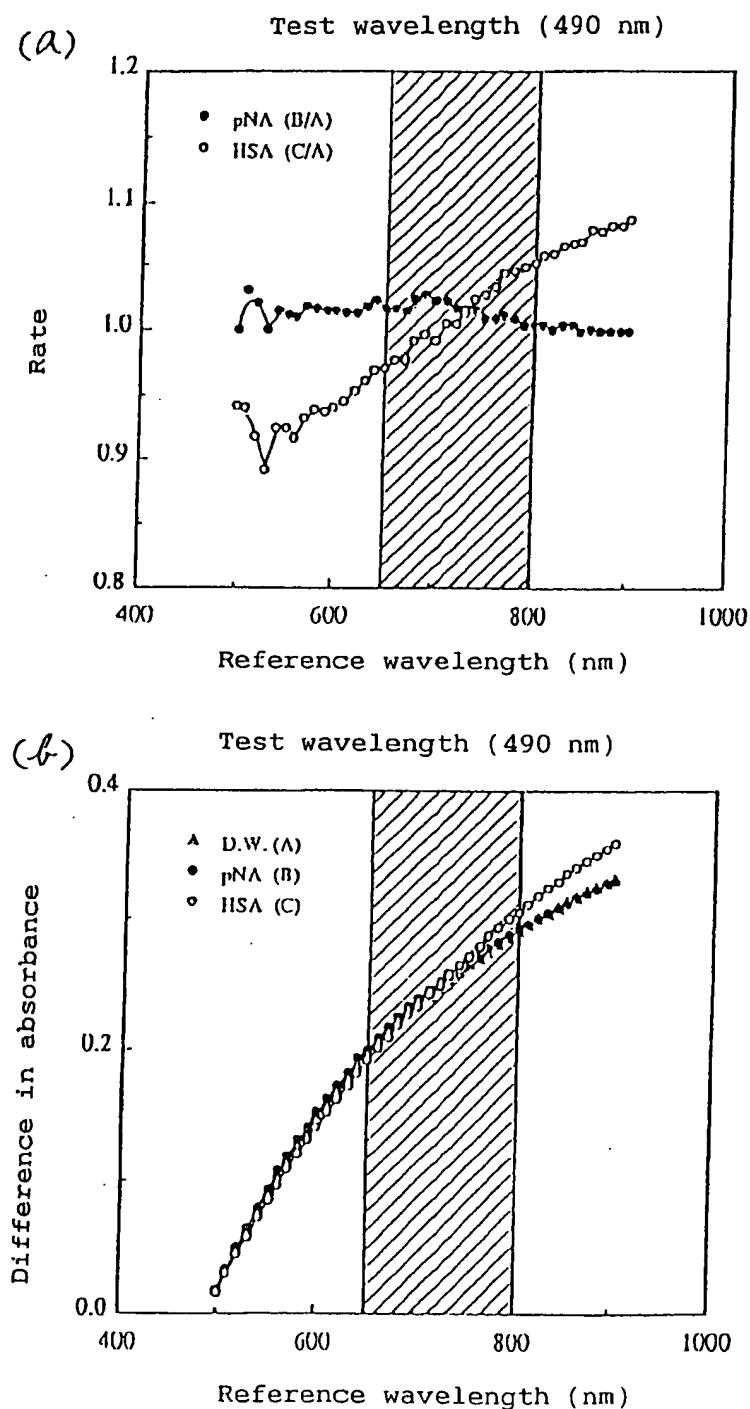
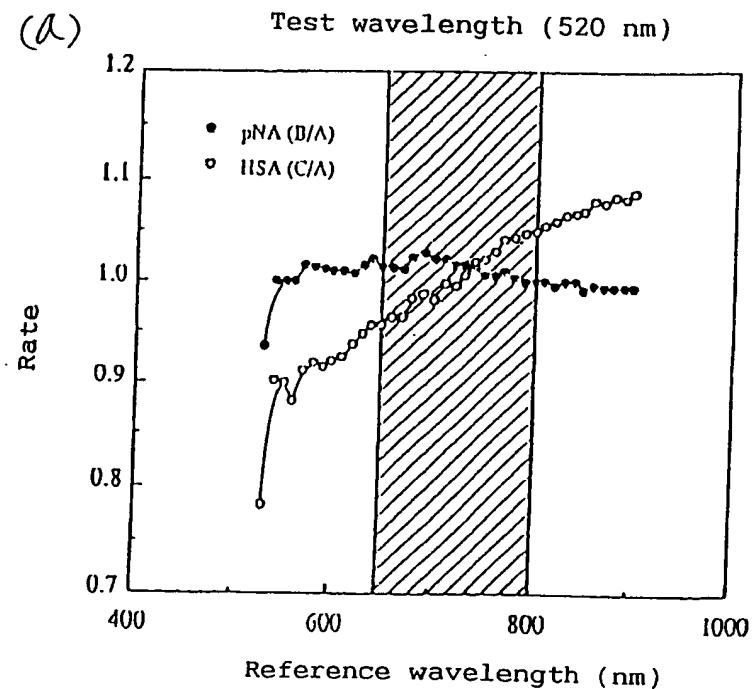


Figure 3

(a)



(b)

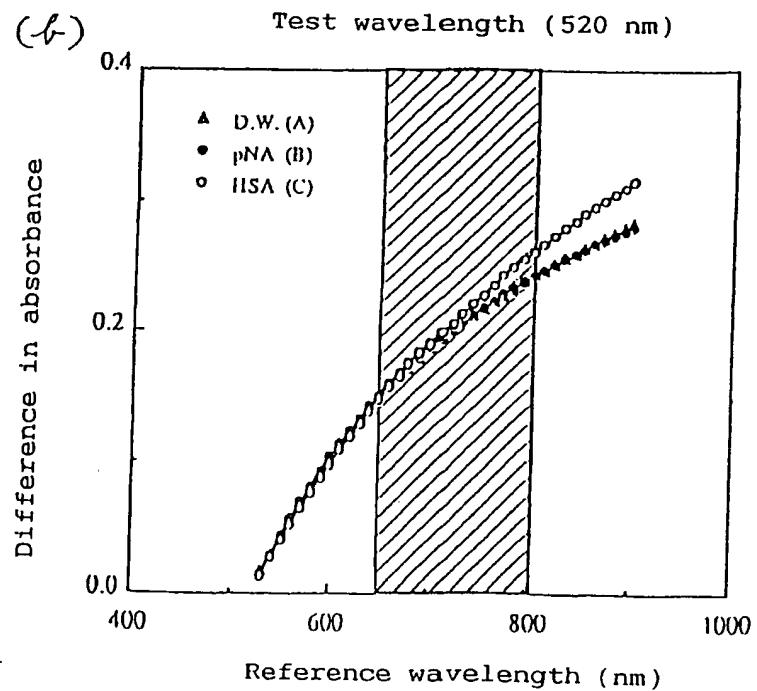


Figure 5

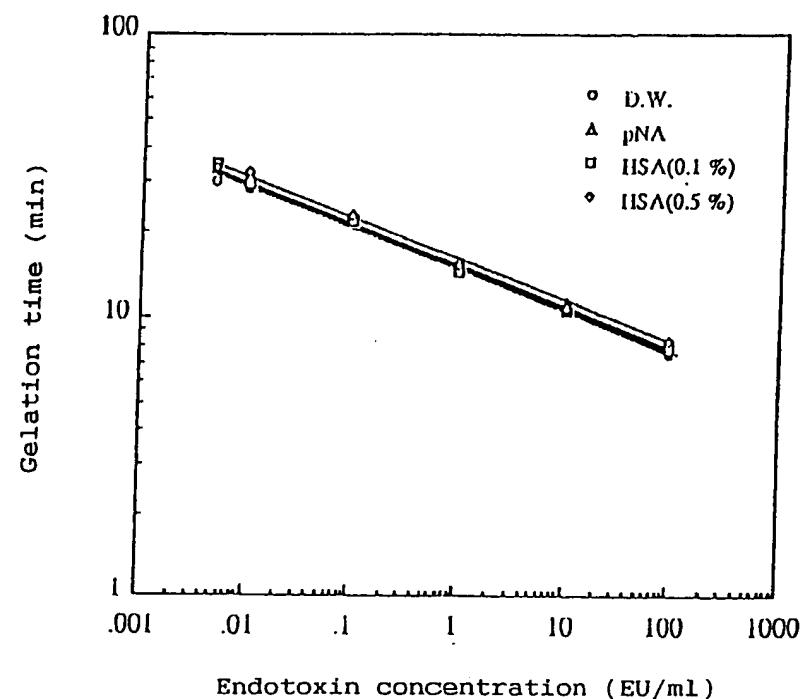
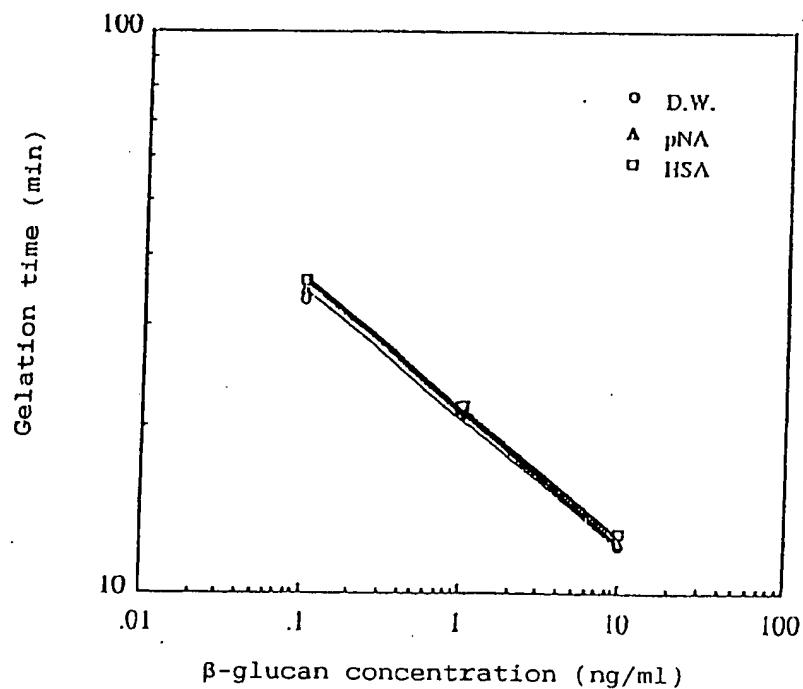


Figure 6



<b>INTERNATIONAL SEARCH REPORT</b>		International application No. PCT/JP94/01973
<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl <sup>6</sup> G01N33/579 According to International Patent Classification (IPC) or to both national classification and IPC		
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Jitsuyo Shinan Koho 1926 - 1994 Kokai Jitsuyo Shinan Koho 1971 - 1994		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, A, 52-90990 (Walsington Biochemical Corp.), July 30, 1977 (30. 07. 77) & US, A, 4038029	1, 3
A	JP, A, 58-9050 (Organo Japan, Ltd.), January 19, 1983 (19. 01. 83), (Family: none)	1-3
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
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Date of the actual completion of the international search  January 19, 1995 (19. 01. 95)	Date of mailing of the international search report  February 7, 1995 (07. 02. 95)	
Name and mailing address of the ISA/  Japanese Patent Office Facsimile No.	Authorized officer  Telephone No.	

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(54) Title : METHOD OF ASSAYING LIMULUS REAGENT-REACTIVE SUBSTANCE

(54) 発明の名称 リムルス試薬反応性物質の測定法

(57) Abstract

A method of determining the concentration of Limulus reagent-reactive substance (endotoxin or (1→3)- $\beta$ -D-glucan) in a specimen by the turbidimetric Limulus test without being affected by any interfering substance with high sensitivity and high accuracy, which method comprises treating a specimen solution with a Limulus reagent to cause gelation and irradiating the resulting solution with the rays of light having a wavelength of 340-420 nm to detect the rise of the solution turbidity caused by the gelation.

(57) 要約

比濁法リムルステストによって、リムルス試薬反応性物質を測定する際に、妨害物質の影響を受けることなく、高感度かつ高精度に測定できる測定波長を決定することを目的として、検体溶液にリムルス試薬を作用させ、ゲル化を起こさせてゲル化に伴う反応液の濁度の上昇を検出し、検体中のリムルス試薬反応性物質（エンドトキシンまたは（1→3）-β-D-グルカン）の濃度を測定する測定法において、反応液に照射する光線の波長を340～420 nmに選定した。

情報としての用途のみ

PCTに基づいて公開される国際出願をパンフレット第一頁にPCT加盟国を同定するために使用されるコード

AM アルメニア	EE エストニア	LK スリランカ	RU ロシア連邦
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BF ブルギナ・ファソ	GB イギリス	MC モナコ	SK スロヴァキア共和国
BG ブルガリア	GE グルジア	MD モルドバ	SN セネガル
BJ ベナン	GN ギニア	MG マダガスカル	SZ スワジランド
BR ブラジル	GR ギリシャ	ML マリ	TD チャード
BY ベラルーシ	HU ハンガリー	MN モンゴル	TG チャコ
CA カナダ	IE アイルランド	MR モーリタニア	TJ タジキスタン
CF 中央アフリカ共和国	IS アイスランド	MW マラウイ	TM トルクメニスタン
CG コンゴー	IT イタリー	MX メキシコ	TT トリニダード・トバゴ
CH スイス	JP 日本	NE ニジェール	UA ウクライナ
CI コート・ジボアール	KE ケニア	NL オランダ	UG ウガンダ
CM カメルーン	KG キルギスタン	NO ノルウェー	US 米国
CN 中国	KP 朝鮮民主主義人民共和国	NZ ニュージーランド	UZ ウズベキスタン共和国
CZ チェコ共和国	KR 大韓民国	PL ポーランド	VN ヴィエトナム
DE ドイツ	KZ カザフスタン	PT ポルトガル	
DK デンマーク	LK リヒテンシュタイン	RO ルーマニア	

## 明細書

## リムルス試薬反応性物質の測定法

## 技術分野

この発明は、リムルス・アメボサイト・ライセート試薬（以下、リムルス試薬  
5 という）を用いて、エンドトキシン、（1→3）- $\beta$ -D-グルカン（以下、 $\beta$ -  
グルカンと略称する）などのリムルス試薬反応性物質を測定する測定法に関する。

## 背景技術

10 エンドトキシンが混入した血液、輸液、注射液を体内に注入すると、強い発熱  
やショックなどの副作用を引き起こすことがあるので、輸液、注射液などの医薬品の製造工程においては、エンドトキシンの混入を防ぐために、その都度エンド  
トキシン量を測定しなければならない。

15 リムルス・アメボサイト・ライセートが、エンドトキシンと反応してゲル化する現象が見出され、従来より発熱試験に代わるエンドトキシンの測定法として、この現象を応用した測定法が実施されている。

20 この測定法としては、ゲル化にともなう反応液（検体溶液とリムルス試薬を含有する反応混合液）の濁りの程度を客観的に判定する目的で、反応液に特定の光線を照射し、時間の経過とともに透過光量の減少（吸光度の増加）を検知して、初期値との比率を求め、この比率が一定値に到達するまでの時間をゲル化時間と判定してエンドトキシン濃度測定の指標とする測定法が知られている（反応速度法による比濁法リムルステストの一種；比濁時間分析法；特公平5-31744号公報参照）。

25 このような光学的な測定法においては、光源として波長660nm程度の光線を放射する発光ダイオードが使用され、光量検出器としてフォト・ダイオード、光電セルなどが使用されている。

また、 $\beta$ -グルカンも、リムルス・アメボサイト・ライセートをゲル化させる

ことが知られている。

このような従来の光学的な測定法においては、光源から照射される光線の波長が、ゲル化して行く反応液の透過度または吸光度の検知に適した波長であるか否かについて考慮されていなかった。

5 また、検体溶液中に着色物質、タンパク、核酸等の妨害物質が存在しても、測定値が安定し、かつ、実用に供し得る感度を有することが必要であるにもかかわらず、従来の測定法においては、このような条件を満たしているとは言えなかつた。

そこで、この発明の測定法は、比濁法リムルステストによって、エンドトキシンまたは $\beta$ -グルカン等のリムルス試薬反応性物質を測定する際に、妨害物質の影響を受けることなく、高感度、高精度に測定できる光線の波長を決定することを目的として考えられたものである。

#### 発明の開示

15 この発明は、検体溶液にリムルス試薬を作用させ、ゲル化を起こさせてゲル化に伴う反応液の濁度の上昇を光線を照射することによって検出し、検体中のリムルス試薬反応性物質を測定する測定法において、反応液に照射する光線の波長を340～420nmの範囲から選定された波長とした。

光線の波長は390～410nmの範囲から選定された波長であることが好ま  
20 しい。また、リムルス試薬反応性物質としては、エンドトキシンまたは $\beta$ -グルカンが挙げられる。

#### 図面の簡単な説明

第1図は、この発明のリムルス試薬反応性物質の測定法で使用する濁度測定装置の一例を示す側面図、

第2図は、反応液に照射する光線の波長に対する吸光度値を示す特性曲線図、

第3図は、反応液の吸光度値の変化率と、リムルス試薬反応性物質（エンドトキシン）の濃度との関係を示す特性曲線図、

第4図は、反応液の吸光度値がある閾値に到達するまでの時間と、リムルス試

薬反応性物質（ $\beta$ -グルカン）の濃度との関係を示す特性曲線図である。

#### 発明を実施するための最良の形態

この発明で使用するリムルス試薬とは、リムルス・ポリフェムス (*Limulus polyphemus*)、タキプレウス・トリデンタツス (*Tachypleus tridentatus*)、タキプレウス・ギガス (*T. gigas*)、カルシノスコルピウス・ロツンディカウダ (*Cariniscorpius rotundicauda*) 等のカブトガニ血リンパ液から、通常の方法（例えば、*Journal of Biochemistry*, 80, 1011-1021(1976)参照）により調製した血球抽出物（リムルス・アメボサイト・ライセート）またはその加工物であり、エンドトキシンおよび／または $\beta$ -グルカンがこれに作用してゲル化する試薬である。

上記加工物とは、例えば、リムルス・アメボサイト・ライセートをクロロホルム等の有機溶媒で抽出処理したり、界面活性剤を添加してエンドトキシンに対する感受性を向上させたものが挙げられる。また、リムルス・アメボサイト・ライセートには、通常、エンドトキシン感受性因子（C因子）および $\beta$ -グルカン感受性因子（G因子）の両方が含まれるが、上記加工物としては、デキストラン硫酸、スルホプロピル基等を結合した担体等を用いてリムルス・アメボサイト・ライセートを処理し、上記C因子またはG因子の何れか一方の因子を分画もしくは除去してエンドトキシンまたは $\beta$ -グルカンの一方のみに作用するように加工されたものが挙げられる。

さらに、リムルス・アメボサイト・ライセートに、例えば（1→3）- $\beta$ -D-グルコシド構造単位が特定個数結合したポリグリコシドを共存させることによって、G因子の活性化を阻害し、エンドトキシンにのみ反応するように加工したものも上記加工物に包含される。なお、リムルス試薬は液体、粉末、固形物等のいずれの形態であってもよい。

この発明においてリムルス試薬反応性物質を測定すべき検体溶液は、特に限定されることはなく、血液、尿、髄液、輸液、注射液、水などが例示される。

検体溶液を、必要に応じて前処理（酸処理、アルカリ処理、加熱処理、界面活性剤処理等）し、適当な反応容器中でリムルス試薬を作用させ、ゲル化を起こさ

せてゲル化に伴う濁度の上昇を、反応液に340～420 nmの範囲から選定された波長の光線を照射し、吸光度を測定することによって検出する。そして、既知濃度のリムルス試薬反応性物質を含む溶液について同様に測定して作成した検量線と測定により得られた値とを比較して検体溶液中のリムルス試薬反応性物質を定量することができる。検量線と測定により得られた値との比較は、予め作成されたプログラムに従って自動的に行なうこともできる。

光線の波長が340 nmより小さい場合、および420 nmより大きい場合には、検体溶液中に共存する可能性のある種々の妨害物質の影響を受けてリムルス試薬反応性物質の水溶液と同等の吸光度が得られない。なお、代表的な妨害物質としては280 nmに極大吸収を有するタンパク、260 nmに極大吸収を有する核酸など挙げられる。

検体溶液にリムルス試薬を作用させるには、検体溶液とリムルス試薬を混合して反応液とし、30～50°C、好ましくは35～45°Cにおいて15分～3時間、好ましくは30分～90分反応させればよい。

吸光度の測定方式は、反応を停止した後測定する方式（エンドポイント法）、または反応させながら測定する方式（反応速度法；カイネティック法）の何れであってもよい。反応速度法としては、反応液の吸光度（透過光量）が一定の値に達するまでの時間をゲル化時間とする方式（比濁時間分析法）、反応液の吸光度の変化率を測定する方式などが挙げられる。

なお、反応容器は、通常のリムルステストに使用される容器であればよく、実質的に光の透過に影響を及ぼさない材質および形状の容器が使用される。例えば、ガラス、プラスチック等の試験管またはマイクロプレートが好適である。

この発明を実施するための形態で使用する吸光度測定装置は、例えば、マイクロプレートを反応容器とした場合、第1図に示すように、キャリア5に載せられ、検体および試薬よりなる反応液14を入れる複数のウェル11を設けた測定用マイクロプレート1と、電球などの発光体31、スリット32、選定された特定波長の光線を透過させるフィルタ33よりなる光源3と、このフィルタ33で選定された光線を測定用マイクロプレート1のウェル11の列数に分割する光学纖維34と、反応液14を透過した光線の強度を電気信号に変換する光電変換素子4よりなる光学系を

備えている。なお、マイクロプレート1の底面に設けられた孔21を有するアルミ板2は、温度分布を均一化するためのものである。

[発明を実施するための形態1：エンドポイント法によるエンドトキシンの測定]

5 この発明のリムルス試薬反応性物質の測定法において使用する光線の最適な波長を求めるために、次の実験を行なった。

市販のリムルス試薬（Associates of Cape Cod社製造、生化学工業株式会社が「パイロテル」という名称で販売している試薬）1mLをガラス試験管に採り、  
10 大腸菌O111:B4株由来のエンドトキシン溶液（0.25EU/mL:EU  
は、Endotoxin Unitの略）1mLを加え、37°Cで30分間反応させた後、10  
%トリフルオロ酢酸0.02mLを添加して反応を停止させた。

次いで、

15 (条件A) 上記反応物に対して、タンパクとして25%ヒト血清アルブミン(HSA) 製剤(ミドリ十字株式会社製)を終濃度で0.5%となるように添加し、  
(条件B) 上記反応物に対して、黄色色素として5mMのバラニトロアニリン(pNA)を終濃度で0.5mMとなるように添加し、

(条件C) 上記反応物に対して、蒸留水(DW)を条件Aおよび条件Bの水溶液と同量添加して、

20 3種の検体溶液を作り、これら3種の検体溶液に対して、波長範囲200nm～900nmにおける吸光度値(濁度)を測定した。

それらの結果は、第2図の特性曲線図に示すとおりであり、タンパク共存状態(条件A:HSA)でも、黄色色素共存状態(条件B:pNA)でも、蒸留水(条件C:DW)と同値を示し、かつ、比較的高い吸光度を呈する波長範囲は、波長340～420nmであり、特に好ましい波長範囲は390～410nmの範囲であることが明らかになった。

そこで、この発明の測定法においては、この知見に基づいて波長340～420nmの範囲の光線を使用して吸光度値の測定を行なって、リムルス試薬反応性物質の濃度の指標とするものである。

[発明を実施するための形態2：反応速度法によるエンドトキシンの測定]

市販されているリムルス試薬（Associates of Cape Cod社製造、生化学工業株式会社が「パイロテルーT」という名称で販売している試薬）0.05mlを9  
6穴マイクロプレート（生化学工業株式会社から発売されているトキシペットブ  
レート96F）のウェルに採り、大腸菌O113株由来のエンドトキシン溶液の  
5 0.01~100EU/mlの範囲内で濃度が異なる5種類のもの各0.05ml  
を加えた。それらに対して、

（条件A）タンパクとして25%ヒト血清アルブミン（HSA）製剤を終濃度  
で0.5%となるように添加し、

10 （条件B）黄色色素として5mMのパラニトロアニリン（pNA）を終濃度で  
0.5mMとなるように添加し、

（条件C）蒸留水（DW）を条件A、条件Bの各水溶液と同量添加して、

15種（=5種類×3条件）の反応液を作り、マイクロプレートの各ウエルに  
採り、恒温槽を備えたマイクロプレートリーダー（生化学工業株式会社から発売  
されているウエルリーダーSK601）にセットし、攪拌した後、37°Cで反応  
15 させながら、波長400nmの光線を15秒間隔で照射して、吸光度値を測定し  
た。

そして、上記吸光度値の変化率をエンドトキシンの各濃度に対して、それぞれ  
対数プロットしたところ、第3図に示す特性を得ることができた。

20 第3図の特性曲線図（検量線）より、タンパク共存状態（条件A：HSA）、  
黄色色素共存状態（条件B：pNA）でも、蒸留水（条件C：DW）とほぼ同じ  
吸光度値の変化率を示し、かつ、この変化率が、エンドトキシン濃度と正比例し、  
妨害物質が共存しても良好な直線性および再現性が得られることが明らかになっ  
た。

[発明を実施するための形態3：反応速度法によるβ-グルカンの測定]

25 「パイロテルーT」0.05mlをトキシペットブレート96Fのウェルに採  
り、β-グルカンとしてパキマン溶液（0.1N NaOHに溶解後、蒸留水で  
希釈）の0.01~1ng/mlの範囲内で濃度が異なる3種類のもの各0.05  
mlを加えた。それらに対して、

（条件A）タンパクとして25%ヒト血清アルブミン（HSA）製剤を終濃度

で0.5%となるように添加し、

(条件B) 黄色色素として5mMのパラニトロアニリン(pNA)を終濃度で0.5mMとなるように添加し、

(条件C) 蒸留水(DW)を条件A、条件Bの各水溶液と同量添加して、

5 9種(=3種類×3条件)の反応液を作り、波長410nmの光線を使用したほかは発明を実施するための形態2と同様に操作した。

そして、吸光度値が、一定の閾値(吸光度値が0.020)に到達するまでの時間(ゲル化時間)tを、各 $\beta$ -グルカン濃度に対して、それぞれ対数プロットしたところ、第4図に示す特性を得ることができた。

10 第4図の特性曲線図より、 $\beta$ -グルカンの測定においても、妨害物質の影響を受けることなく、良好な直線性および再現性が得られることが明らかになった。

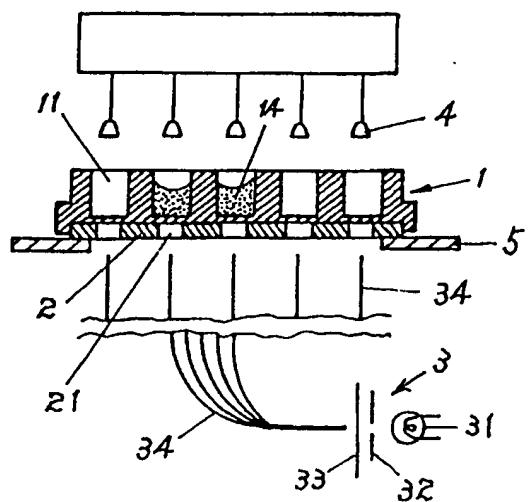
#### 産業上の利用可能性

以上の発明を実施するための形態に基づく説明から明らかなように、この発明のリムルス試薬反応性物質の測定法によると、リムルス試薬反応性物質としてエンドトキシンまたは $\beta$ -グルカンを用いた比濁法リムルステストにより測定する方法において、波長範囲が340~420nm、好ましくは390~410nmの光線を使用することにより、共存タンパクや共存色素の妨害を受けることなく、高感度、高精度で、安定した測定が可能になる。

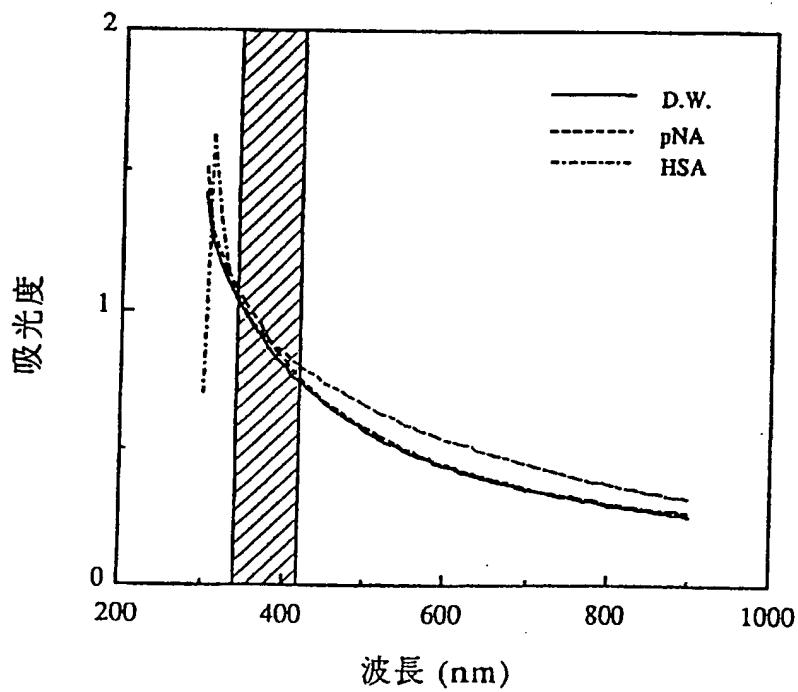
## 請求の範囲

1. 検体溶液にリムルス試薬を作用させ、ゲル化を起こさせてゲル化に伴う反応液の濁度の上昇を光線を照射することによって検出し、検体中のリムルス試薬反応性物質を測定する測定法において、反応液に照射する光線の波長を 340～4  
5 20 nm の範囲から選定された波長としたことを特徴とするリムルス試薬反応性物質の測定法。
2. 光線の波長が 390～410 nm の範囲から選定された波長である請求の範  
10 囲 1 に記載のリムルス試薬反応性物質の測定法。
3. リムルス試薬反応性物質が、エンドトキシンまたは(1→3)- $\beta$ -D-グル  
15 カンであることを特徴とする請求の範囲 1 に記載のリムルス試薬反応性物質の測定法。

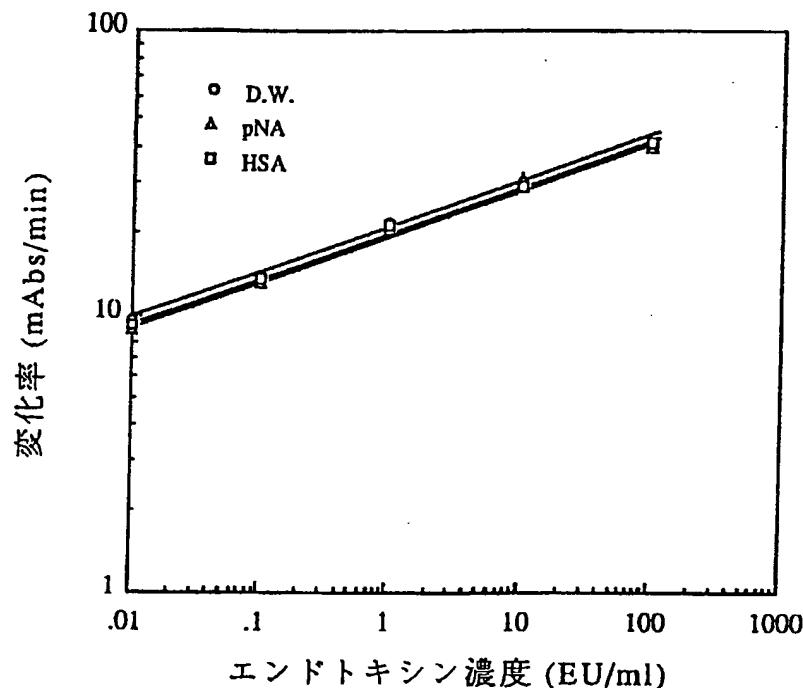
第1図



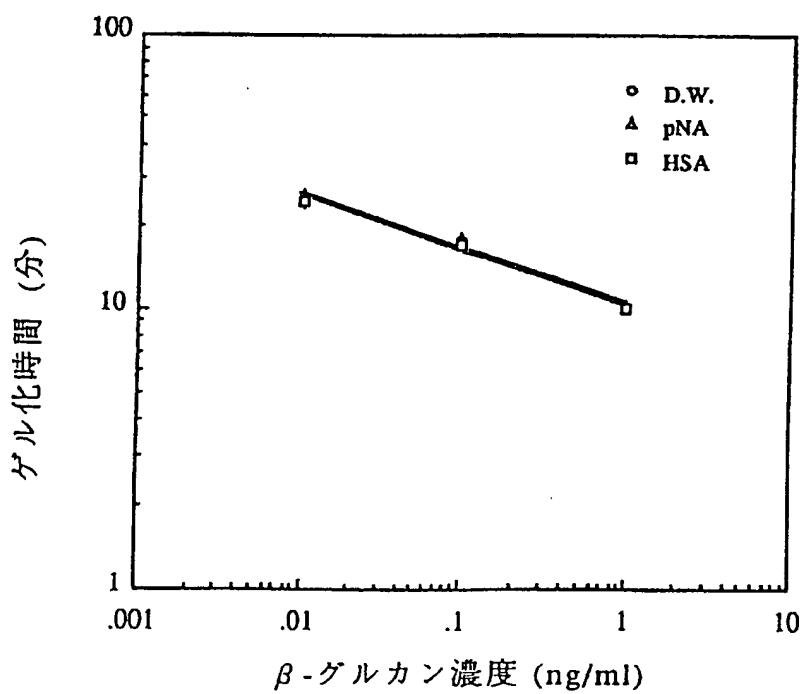
第2図



第3図



第4図



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP94/01973

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl<sup>6</sup> G01N33/579

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl<sup>6</sup> G01N33/579

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1926 - 1994  
 Kokai Jitsuyo Shinan Koho 1971 - 1994

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, A, 52-90990 (Warsington Biochemical Corp.), July 30, 1977 (30. 07. 77) & US, A, 4038029	1, 3
Y		2
A	JP, A, 58-9050 (Organo Japan, Ltd.), January 19, 1983 (19. 01. 83), (Family: none)	1-3

<input type="checkbox"/> Further documents are listed in the continuation of Box C.	<input type="checkbox"/> See patent family annex.
Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance
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"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search  January 19, 1995 (19. 01. 95)	Date of mailing of the international search report  February 7, 1995 (07. 02. 95)
Name and mailing address of the ISA/  Japanese Patent Office Facsimile No.	Authorized officer  Telephone No.

## 国際調査報告

国際出願番号 PCT/JP 94/01973

## A. 発明の属する分野の分類(国際特許分類(IPC))

Int. CL<sup>6</sup> G 01 N 33/579

## B. 調査を行った分野

## 調査を行った最小限資料(国際特許分類(IPC))

Int. CL<sup>6</sup> G 01 N 33/579

## 最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報 1926-1994年

日本国公開実用新案公報 1971-1994年

## 国際調査で使用した電子データベース(データベースの名称、調査に使用した用語)

## C. 関連すると認められる文献

引用文献の カテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する 請求の範囲の番号
X Y	J P, A, 52-90990 (ワースイングトン・バイオケミカル コーポレーション), 30. 7月. 1977 (30. 07. 77) & U S, A, 4038029	1, 3 2
A	J P, A, 58-9050 (オルガノ株式会社), 19. 1月. 1983 (19. 01. 83) (ファミリーなし)	1-3

 C欄の続きにも文献が列挙されている。 パテントファミリーに関する別紙を参照。

## \* 引用文献のカテゴリー

「A」特に関連のある文献ではなく、一般的技術水準を示すもの  
 「E」先行文献ではあるが、国際出願日以後に公表されたもの  
 「I」優先権主張に疑義を提起する文献又は他の文献の発行日  
 若しくは他の特別な理由を確立するために引用する文献  
 (理由を付す)  
 「O」口頭による開示、使用、展示等に言及する文献  
 「P」国際出願日前で、かつ優先権の主張の基礎となる出願の日  
 の後に公表された文献

「T」国際出願日又は優先日後に公表された文献であって出願と  
 矛盾するものではなく、発明の原理又は理論の理解のために  
 引用するもの

「X」特に関連のある文献であって、当該文献のみで発明の新規性  
 又は進歩性がないと考えられるもの

「Y」特に関連のある文献であって、当該文献と他の1以上の文  
 献との、当業者にとって自明である組合せによって進歩性  
 がないと考えられるもの

「&」同一パテントファミリー文献

国際調査を完了した日  19. 01. 95	国際調査報告の発送日  07.02.95
名称及びあて先  日本国特許庁 (ISA/JP) 郵便番号100 東京都千代田区霞が関三丁目4番3号	特許庁審査官(権限のある職員)  龟田 宏之 電話番号 03-3581-1101 内線 3250